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CHANGES IN TETANIC AND RESTING $[Ca^{2+}]_i$ DURING FATIGUE AND RECOVERY OF SINGLE MUSCLE FIBRES FROM *XENOPUS LAEVIS*

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SUMMARY

1. Single muscle fibres were dissected from the toe muscles of *Xenopus laevis* and microinjected with Fura-2 to measure myoplasmic calcium concentration ($[Ca^{2+}]_i$). Injected fibres were illuminated at 340 and 380 nm and the ratio of the resulting fluorescence at 505 nm (the Fura-2 ratio) was taken as a measure of $[Ca^{2+}]_i$. Fibres were fatigued at 21 °C by repeated tetani until developed tension had fallen to 50% of control.

2. Tetanic tension declined monotonically during fatiguing stimulation, whereas the tetanic Fura-2 ratio first increased and then declined. At the 10th tetanus, tension was 87% of control whereas the Fura-2 ratio was 106% of control. At the end of fatiguing stimulation, where tension was around 50% of control, the tetanic Fura-2 ratio was reduced to 71%. The rate of decline of both tension and the Fura-2 ratio after a tetanus slowed during fatigue. During recovery, the tension and the tetanic Fura-2 ratio recovered in parallel.

3. The resting Fura-2 ratio increased throughout fatigue reaching 237% of control when tension had declined to 50%. There was a rapid phase of recovery, complete within 1 min, by which time the resting Fura-2 ratio was 198% of control. Subsequent recovery was slower and took 20–30 min to reach a stable level which was 121% of control.

4. The resting Fura-2 ratio towards the end of fatiguing stimulation was greater than the tetanic Fura-2 ratio in the early part of recovery although there was no detectable increase of resting tension during fatiguing stimulation. This observation suggests that the Ca^{2+} sensitivity of the contractile proteins was reduced at the end of fatiguing stimulation.

5. Plots of the tetanic tension against tetanic Fura-2 ratios throughout fatiguing stimulation and recovery also suggested that Ca^{2+} sensitivity was reduced during fatiguing stimulation when compared to recovery.

6. The increases in resting $[Ca^{2+}]_i$ caused by raised $[K^+]_o$ (from 2·5 to 10 mM) and/or by application of 15% CO_2 were much less than those produced by fatiguing

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stimulation. Much of the elevated $[Ca^{2+}]_i$ in fatigue could be reversed by application of dantrolene (25 μM).

7. The results suggest that both reduced tetanic $[Ca^{2+}]_i$ and reduced Ca^{2+} sensitivity contribute to the decline of tension during fatigue. The elevated resting $[Ca^{2+}]_i$ during fatigue appears to result from either an increase in the Ca^{2+} leak from the sarcoplasmic reticulum or a reduction in Ca^{2+} reuptake by the sarcoplasmic reticulum.

INTRODUCTION

Incomplete activation of the contractile proteins may contribute to the tension reduction associated with skeletal muscle fatigue (Eberstein & Sandow, 1963; Grabowski, Lobsiger & Lüttgau, 1972; Lännergren & Westerblad, 1989). Incomplete activation could be due either to reduced Ca^{2+} release from the sarcoplasmic reticulum (SR) and/or to reduced Ca^{2+} sensitivity of the contractile proteins. In support of the first possibility, we have recently shown that most of the tension reduction in fatigued *Xenopus* muscle fibres can be explained by reduced myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) during tetani (Allen, Lee & Westerblad, 1989*b*). We also showed preliminary evidence for a rise in resting $[Ca^{2+}]_i$ in fatigued fibres. Such an increase in resting $[Ca^{2+}]_i$ might be of great physiological importance by activating enzyme systems (e.g. Solandt, 1936; Duncan, 1978) and by causing structural damage (e.g. Chapman & Tunstall, 1987).

In our study quoted above, the Ca^{2+}-sensitive photoprotein aequorin was microinjected into intact, single muscle fibres and fatigue was produced by repeated tetani. We have now performed similar experiments using the fluorescent Ca^{2+} indicator Fura-2 (Grynkiewicz, Poenie & Tsien, 1985). Compared with aequorin, Fura-2 has the advantage that it responds linearly to low $[Ca^{2+}]$ and gives a readily measurable signal at the $[Ca^{2+}]_i$ found in resting muscle fibres. Further, it is little affected by changes in the concentration of magnesium ions or hydrogen ions (Grynkiewicz *et al.* 1985). Thus, changes in the resting $[Ca^{2+}]_i$ are probably more easily and reliably detected with Fura-2 than with aequorin. The present results demonstrate changes of the tetanic $[Ca^{2+}]_i$ during fatigue which are similar to those obtained with aequorin, but indicate in addition that a reduced Ca^{2+} sensitivity may explain some of the tension reduction in fatigued fibres. The results also show that a marked increase of the resting $[Ca^{2+}]_i$ develops during fatiguing stimulation and that this increase takes about 20–30 min to recover. Some of these results have been presented in abstract form (Allen, Lee & Westerblad, 1989*a*).

METHODS

Fibre dissection, mounting and stimulation

Adult, female *Xenopus laevis* were killed by decapitation and the spinal cord destroyed. Single fibres were dissected from any of the lumbrical muscles II–IV of the foot. After measurement of fibre dimensions, platinum micro-clips were attached to the tendons and the preparation transferred to the perfusion channel of the experimental chamber. The fibre was mounted between a force transducer (Akers AE 801, SensorNor, Norway) and an adjustable holder, allowing the fibre to be stretched to the length which gave maximum tetanic tension. The fibre was flanked by platinum electrodes and stimulated by current pulses with a duration of 0·5 ms and an intensity

of about 1·2 × threshold. Fibres were superfused at room temperature (21 °C) by a Ringer solution of the following composition (mM): Na^+, 120; K^+, 2·5; Ca^{2+}, 1·8; Cl^-, 121; HPO_4^{2-}, 2·15; $H_2PO_4^-$, 0·85; the pH was 7·0.

Injection of Fura-2

Fura-2 was dissolved in 150 mM-KCl (pH 7·0) at a concentration of 10 mM. About 1 μl of this solution was introduced into the back end of a conventional fibre-filled micropipette (resistance 10–40 MΩ). The pipette was placed in a holder which allowed pressure to be applied to the back of the pipette. Patency was checked by placing the tip of the microelectrode in the solution and observing the tip with the microscope in fluorescence mode (see below); patent electrodes showed a burst of fluorescence close to the tip when pressure was applied. Once a patent electrode had been obtained the muscle fibre was penetrated and membrane potential monitored. Brief pulses of pressure were then applied in order to inject Fura-2 into the muscle fibre. It was found that pipettes which were patent when outside the fibre both before and after penetration frequently failed to inject when inside the fibre, as if some aspect of penetration or of the intracellular environment temporarily plugged the tip. Sometimes this plugging could be overcome by increasing the applied pressure or by withdrawing the electrode slightly whilst remaining intracellular.

As a test of whether injected Fura-2 was buffering $[Ca^{2+}]_i$ significantly, twitches were recorded at a fast paper speed both before and after injection. No obvious change of amplitude or time course was observed even with the largest of the injections used in this study, suggesting that little significant buffering was occurring (see also below).

Fluorescence measurements

Fura-2 was used in the 'ratio' manner described by Grynkiewicz *et al.* (1985), i.e. it was illuminated at 340 and 380 nm and the resulting fluorescence signals emitted at 505 nm were divided to obtain a $[Ca^{2+}]$-dependent signal. The optical arrangements are illustrated in Fig. 1*A*. The experiments were performed on the stage (MS) of a Nikon Diaphot microscope with a ×10 Fluor objective (O). The muscle fibre was suspended horizontally in the muscle chamber (MC). For conventional microscopy the fibre was illuminated from above (LS2) through an extra-long working length condenser fitted with a 650 nm long-pass (red) filter (LPF). In fluorescence mode an Ealing Beck 150 W light source with a xenon arc lamp provided the UV illumination (UV LS). A shutter (S) prevented illumination of the preparation except as required and an automated device inserted a 340 (±5) nm or 380 (±5) nm interference filter (IF1 and 2) into the light path. Neutral density filters (NDF) could be added to reduce the intensity of UV illumination. UV illumination of the preparation was via a dichroic mirror (DM1) with a cut-off at 430 nm, so that only shorter wavelengths illuminated the specimen and only longer wavelengths produced by Fura-2 fluorescence or the microscope light source (LS2) were transmitted to the eyepieces (E) and to the measurement port. The measurement port contained a second dichroic mirror (DM2) with a cut-off at 550 nm. Red light from the microscope light source was focused directly on the input of a video camera so that the fibre could be viewed on a monitor throughout the experiment and during fluorescence measurements. Light of shorter wavelengths, emitted by Fura-2 in the fibre, was reflected by this dichroic mirror through a fibre-optic light guide to a photomultiplier tube (PMT) with a 505 (±5) nm interference filter (IF3) in front of its photocathode. The amplified output of this PMT (measured in nA or μA of photocathode current) therefore represents the 505 nm output of the fibre. Changes in the red illumination of the fibre had no perceptible effect on this signal, but it was necessary to exclude room light from the stage and eyepieces.

Three sources of light contributed to this signal at 505 nm: (i) background, i.e. light recorded with the UV illumination on, but no muscle fibre in the light path (fibre-out). This component was substantial (5–20 % of the signal) and was determined at intervals throughout each experiment, by moving the muscle fibre sideways out of the UV beam. All measurements of resting and tetanic Fura-2 signals were made from this fibre-out level, effectively substracting the background signal. (ii) Autofluorescence due to endogenous compounds present in the fibre before injection, e.g. NADH. This was measured in three fibres and averaged 60 nA at 340 nm (range 15–100 nA) and 80 nA at 380 nm (range 30–150 nA). In one experiment autofluorescence was measured throughout a period of fatiguing stimulation but showed no significant changes at either illumination wavelength. No correction was made for this component. (iii) Fluorescent signal from injected Fura-2. This varied according to the size of injection and averaged 5000 nA at 340 nm (range

300–25000 nA) and 16000 nA at 380 nm (range 1000–84000 nA). Thus on average autofluorescence was only 0·5–1·2% of the Fura-2 fluorescence and could be ignored.

In order to determine the injected Fura-2 concentration within the fibres, we compared the above fluorescent signals to those produced by known concentrations of Fura-2 in a glass capillary

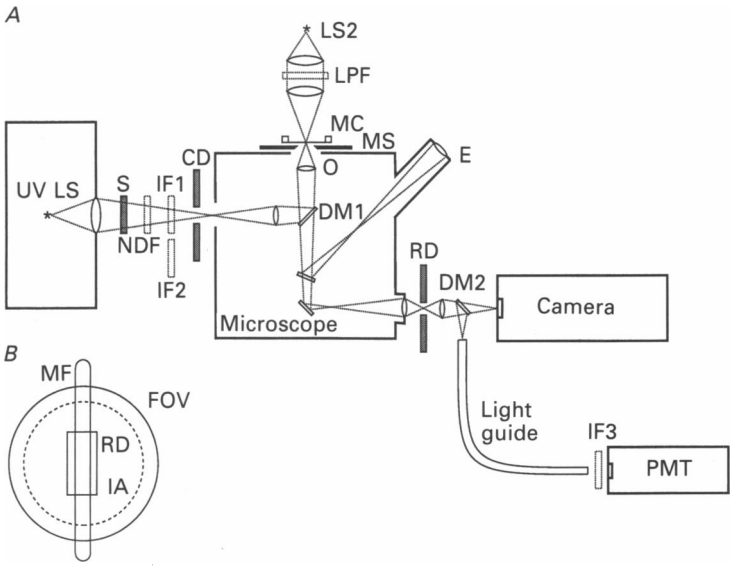

Fig. 1. Diagram of the apparatus. Panel *A* shows the optical arrangement schematically. Panel *B* shows the region of the muscle fibre from which Fura-2 fluorescence was recorded. Panel *A*: UV LS, ultraviolet light source; S, shutter; NDF, neutral density filter; IF, interference filter; CD, circular diaphragm; LS2, tungsten light source; LPF, long-pass filter; MC, muscle chamber; MS, microscope stage; O, objective; DM1 and 2, dichroic mirrors; E, eyepiece; RD, rectangular diaphragm; PMT, photomultiplier tube. Panel *B*: MF, muscle fibre; FOV, field of view of microscope; IA, area illuminated by UV light source; RD, rectangular diaphragm.

with the same internal dimensions as a fibre (Klein, Simon, Szucs & Schneider, 1988). Such measurements indicated that the average fibre contained 7 μM-Fura-2 (range 0·5–35 μM). This concentration is much smaller than endogenous cellular buffers such as troponin and parvalbumin and suggests that Ca^{2+} buffering by Fura-2 should be minimal (cf. Baylor & Hollingworth, 1988).

The ×10 objective used in these studies displayed about two-thirds of the muscle fibre (MF) (total length about 1·5 mm) within the microscope field of view (FOV) (Fig. 1*B*). The UV lamp was adjusted so that more or less even UV illumination was obtained and the illuminated area (IA) was restricted using a circular diaphragm (CD) to about three-quarters of the field of view. Then a rectangular diaphragm (RD) in the measurement port was rotated so that it was aligned with the fibre, and light to the PMT (and camera) was restricted to a region about 50% greater than the width of the fibre and including about one-third of the total length of the fibre.

Experimental protocol

After injection of Fura-2, fibres were left for at least 1 h before being stimulated. This resting period was introduced to allow the dye to diffuse throughout the fibre from the place of injection. After 1 h the fluorescent light was somewhat bigger at the site of injection than at the ends of the fibre. However, in the region of the muscle within the rectangular diaphragm, the gradient of Fura-2 concentration was very small.

After the resting period fibres were (a) fatigued or (b) exposed to CO_2 and/or increased KCl in the rested state.

(a) Fatigue was produced by repeated tetanic contractions following a scheme similar to that

described by Westerblad & Lännergren (1986). Initially 500 ms, 50 Hz tetanic were given every 4·0 s and the inter-tetanus interval was then reduced after 2 min to 3·0 s and after a further 2 min to 2·5 s. Stimulation was continued until tension was reduced to about 50 % of the control. During fatiguing stimulation the illuminating wavelength was changed from 340 to 380 nm at suitable intervals and the Fura-2 ratios shown were calculated from pairs of neighbouring tetani. After fatiguing stimulation recovery of tension and Fura-2 ratios were monitored in some fibres. Pairs of test tetani separated by 1 min were then given after 1, 5, 10, 20, 30 and 40 min of recovery and thereafter every 20 min.

For simplicity, the Fura-2 signal is described by the first of the pair of tetani used for its determination, i.e. the Fura-2 signal described as the 10th contraction of a fatigue run was calculated from the 10th and 11th tetanus, one being illuminated at 340 nm, the other at 380 nm.

(b) These experiments were performed to study the effects of intracellular acidification and depolarization on resting $[Ca^{2+}]_i$. The illuminating wavelength was switched automatically between 340 and 380 nm usually at 4 s intervals. The emitted light was passed to two sample-and-hold circuits so that the signal produced by illumination at each wavelength was held during the period when the other illuminating wavelength was in use. The two continuous signals obtained in this way were passed to an analog divide circuit so that a continuous ratio signal was available, although its time resolution was limited to approximately 4 s.

Interpretation of Fura-2 signals

The Fura-2 records in this paper are presented as the ratio of 340/380 nm signals. These have not been routinely converted to $[Ca^{2+}]_i$ for several reasons (but see Discussion and Fig. 10). It is known that the fluorescent properties of Fura-2 are modified in the intracellular environment (Baylor & Hollingworth, 1988; Konishi, Olson, Hollingworth & Baylor, 1988; Poenie, 1990). That this was happening in the present experiments was clear from the following data. When samples of Fura-2 in 157 mM-K^+, pH 7·0, were illuminated on the stage of the microscope in exactly the same manner as the muscle fibre, high Ca^{2+} (4 mM) gave a ratio (R_{max}) of 8·2, while low Ca^{2+} (EGTA, no added Ca^{2+}) gave a ratio of 0·23 (R_{min}). This gives an $R_{max}/R_{min} = 36$, which is similar to the value obtained by Grynkiewicz *et al.* (1985). However the greatest R_{max} recorded in a muscle was around 3, although we have evidence (see Fig. 4) that Fura-2 was close to saturation with Ca^{2+} under these conditions.

A potentially more serious problem was that the properties of injected Fura-2 appeared to change with prolonged and intense illumination. Similar problems have been observed in other tissues (Becker & Fay, 1987). This problem was mainly observed when resting Ca^{2+} was being measured, because the fibre was then illuminated in one region for a relatively long period. During long illuminations at high light intensities the emitted fluorescence at both 340 and 380 nm gradually declined but, since the 380 nm signal declined more quickly than the 340 nm signal, the resting ratio gradually increased while the peak tetanic ratios declined. Thus, in one fibre at the start of the experiment, before any substantial illumination, the resting ratio was 0·31 and the peak tetanic ratio was 2·31. The fibre was then continuously illuminated for about 30 min (light intensity reduced tenfold by a neutral density filter) and at the end of this period the resting ratio was 0·44 and the peak tetanic ratio 0·79. The developed tension at this time was normal despite the very low Fura-2 ratio and the fibre contracted uniformly suggesting that the underlying $[Ca^{2+}]_i$ was unchanged. In support of this notion, measurements at the other end of the fibre, which had not been continuously illuminated, revealed ratios similar to the original (resting ratio 0·33, peak ratio 2·60). In subsequent experiments these effects were minimized by substantially reducing the intensity and duration of the UV illumination. None of the experiments reported in the paper was performed under conditions which produced these changes in Fura-2 properties (with the exception of Fig. 4*A*). As a further precaution, one experiment was performed in which only every 5th tetanus was illuminated and the intensity of illumination was reduced tenfold. This experiment gave similar results to all others despite a 50-fold reduction in illumination.

Calibration of Fura-2

In order to determine whether the properties of Fura-2 were affected by the intracellular changes which occur during fatigue, the following *in vitro* calibration experiments were performed. The solutions used were designed to mimic the major changes in metabolic compounds which are known to occur in fatigue. Since it is known that the ionic effects of Na^+ and K^+ on Fura-2 are similar

(Williams & Fay, 1990) no attempt was made to produce appropriate proportions of $[K^+]$ to $[Na^+]$ in the solutions.

Solution (i) contained (mM): K^+, 130; Na^+, 27; phosphocreatine, 10; ATP, 3; HEPES, 7; EGTA, and/or Ca^{2+}, 7; Fura-2, 5×10^{-3}; pH was adjusted to 7·0 in the final solution; $\Sigma([K^+]+[Na^+]) =$ 157 mM. $[Ca^{2+}]$ was set by EGTA buffers to high Ca^{2+} (4 mM-Ca^{2+} + 3 mM-CaEGTA), low Ca^{2+} (7 mM-EGTA) and three solutions were made with known ratios of CaEGTA to EGTA and the $[Ca^{2+}]$ calculated using the EGTA binding constants of Schwarzenbach, Senn & Anderegg (1957) (apparent dissociation constant at pH 7·0 = 360 mM). This solution gave $R_{max} = 8{\cdot}17$ (range 8·1–8·2, $n = 3$), $R_{min} = 0{\cdot}23$ (range 0·22–0·24) and $K_d = 283$ nM (range 250–325). This K_d is similar to the value determined by Williams & Fay (1990) (250 nM) at the same $\Sigma([Na^+]+[K^+])$.

Solution (ii) contained (mM): K^+, 144; Na^+ 20; creatine, 10; PO_4^{2-}, 19; lactate, 13; HEPES, 7; EGTA, 7; pH 6·50; $\Sigma([K^+]+[Na^+]) = 164$ mM. The EGTA dissociation constant at pH 6·50 is 3550 nM (Schwartzenbach *et al.* 1957). In this solution $R_{max} = 8{\cdot}32$ (range 8·30–8·35, $n = 3$), $R_{min} =$ 0·23 (range 0·21–0·24) and $K_d = 366$ nM (range 350–400). Thus, changes in R_{max} and R_{min} between the two solutions are very small but there is a fall in apparent Ca^{2+} sensitivity of Fura-2 which is possibly due to the acidosis (note the much larger change in EGTA binding constant over the same pH range). If these changes in Fura-2 affinity for Ca^{2+} were to occur *in vitro*, they could account for some of the decline in peak tetanic fluorescence in fatigue and would lead to an underestimate of the Ca^{2+} desensitization which was observed (see Results). However, the true situation within the muscle will also be affected by the changes in $\Sigma([K^+]+[Na^+])$ (Williams & Fay, 1990). Juel (1986) has measured $[K^+]_i$ and $[Na^+]_i$ in control and fatigued muscles and the $\Sigma([K^+]_i+[Na^+]_i)$ falls from 181 mM in control to 160 mM in fatigue, which would reduce the increase in K_d back to approximately the control level.

In summary, it appears that the changes in metabolites lead to a reduction in the sensitivity of Fura-2 to Ca^{2+}. The increase in K_d we observed in 'fatigue' conditions *in vitro* is probably counteracted by the changes in $\Sigma([K^+]_i+[Na^+]_i)$ occurring in fatigue. Thus it seems unlikely that changed Fura-2 properties due to changes of the intracellular milieu in fatigue made any important contribution to our results.

Movement artifacts

Fibres moved during the rapid rise of tension at the onset of contraction due to movements of the platinum clips, stretching of tendon insertions, etc.; during relaxation the same type of movements occurred and in addition longitudinal movements were observed (see Huxley & Simmons, 1970). In principle, the ratio method eliminates artifacts due to movements because they affect both wavelengths equally. However, in our system, where the two illuminating wavelengths are applied to different tetani, the correction will not be perfect if movements are not identical in the two successive tetani used for measurement. To minimize movement artifacts, the section of the fibre within the rectangular diaphragm (Fig. 1*B*), from which the measurements were made, was displayed on a video screen throughout experiments, and we have excluded measurements of tetanic ratios from experiments where the fibre approached or moved out of the rectangular diaphragm. Further, measurements have only been done from two successive tetani where the tension records were close to identical, which indicates the movements to be similar.

During stimulation (after the tension rise) no movement was observed except in tetani which were not 'flat-topped', i.e. showed a slow increase ('creep') or decrease ('sag') of tension. The movements associated with creep during fatigue were small (see also Fig. 3, Curtin & Edman, 1989), and we consider it unlikely that they would influence tetanic ratios. Somewhat larger movements were observed in tetani displaying sag, and these movements coincided with the tension decline (see also Westerblad & Lännergren, 1990). We have here measured peak tetanic ratios, which generally were obtained at the time of maximum tension and thus before the movements associated with sag occurred.

In summary, it is unlikely that movement artifacts significantly influenced measurements of the peak tetanic ratio. Ratio signals from the tension rise and the relaxation of tetani, on the other hand, might have been affected by movements.

Statistics

All statistical data are presented as mean and S.E.M. and the number of observations is given. A total of twelve fibres were used in these experiments but no fibres completed all the experiments described.

RESULTS

Fura-2 signals before fatiguing stimulation

Figure 2 shows the main features of the Fura-2 signal during a short tetanus. Three tetani at 4 min intervals were produced and the fibre was illuminated at 340, 360 and

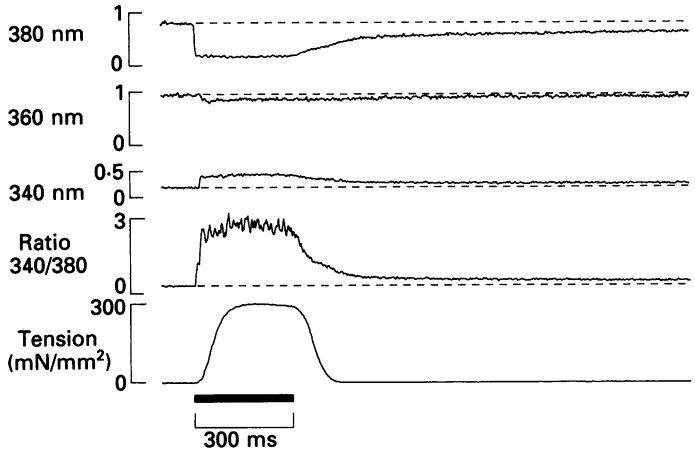

Fig. 2. Fluorescence signals and tension during a brief tetanus. Three upper traces show fluorescence signals recorded at 505 nm when the fibre was illuminated at 380, 360 and 340 nm in three successive tetani. Fourth trace shows the calculated 340/380 ratio signal. Tension record is the average of the three tetani used to record the fluorescence signals. Bar shows duration of stimulation.

380 nm. Grynkiewicz *et al.* (1985) have demonstrated that 360 nm is approximately the isosbestic point for Fura-2 *in vitro*. The *in vivo* records during illumination at 360 nm show a small (10–15%) reduction in fluorescence during stimulation which probably represents the fact that the isosbestic point is not exactly 360 nm (e.g. Klein *et al.* 1988) though there could, in addition, be a very small movement artifact. The signals at 340 and 380 nm were close to mirror images of each other; both showed a rapid early change to a more or less steady level during the tetanus and then a complex recovery during relaxation. The ratio signal shows the results of dividing the 340 nm by the 380 nm signal. This ratio is much less sensitive to movement artifacts than the signal for individual wavelengths (see Methods) and it also is independent of the amount of Fura-2 injected. The ratio signal exhibits three phases as it recovers following a tetanus. Shortly after the last stimulus the signal began to decline rapidly (phase I). This decline continued until the ratio was between 75 and 50% of the maximum and then the rate of decline showed a marked reduction, which was sometimes so large that the ratio rose briefly (phase II). This phase is clearer in Fig. 3, which shows the relaxation phase of a 2 s tetanus in more detail. Finally (phase III), when tension had declined to zero, the ratio fell very slowly back to the control. The initial half-time of this fall was $3{\cdot}2 \pm 0{\cdot}25$ s ($n = 8$) and subsequent half-times were longer. Elevation of resting $[Ca^{2+}]_i$ was generally undetectable after 30 s. Similar features have been shown with aequorin (Cannell, 1986) but, because of the non-linear relation between light and $[Ca^{2+}]_i$ for aequorin, the last two phases represent only 1–2% of the signal and are therefore difficult to observe.

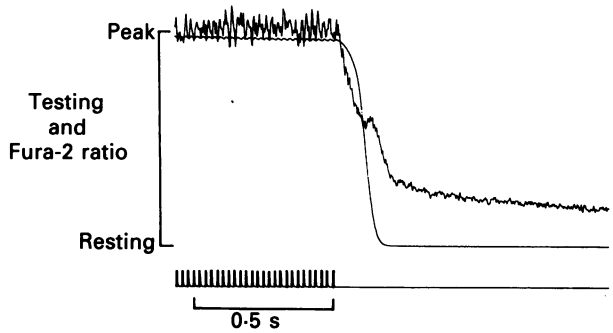

Fig. 3. Fura-2 ratio signal and tension at the end of a 2 s tetanus. Peak and resting level of both traces superimposed to facilitate comparison of the time course of recovery of the two signals.

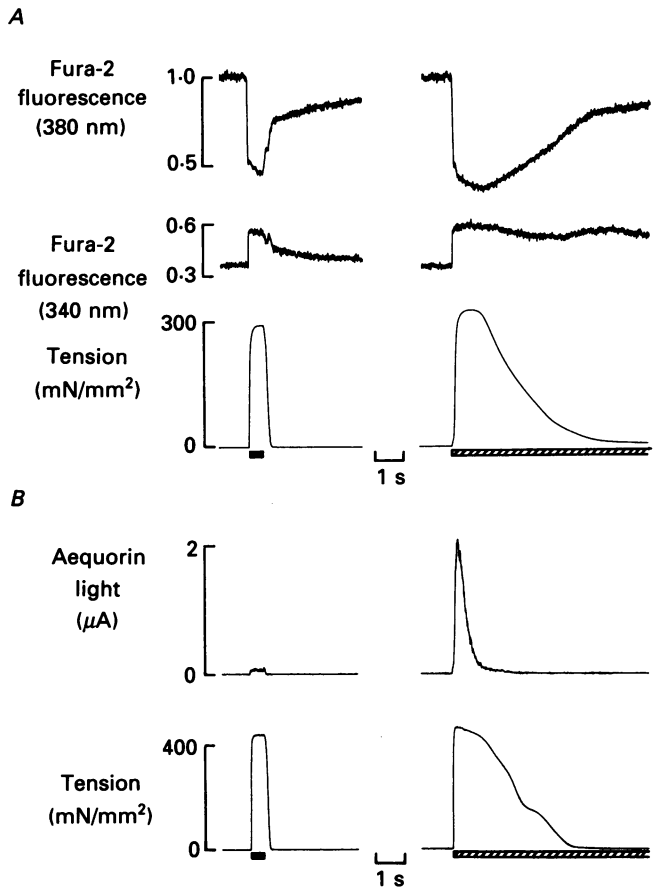

Fig. 4. Comparison of Fura-2 and aequorin signals during tetani and K^+ contractures. Panel *A*, Fura-2-injected fibre. Left-hand panel shows a 0·5 s tetanus; right-hand panel shows a contracture produced by 150 mM-K^+ in the same fibre. Upper two traces show Fura-2 fluorescence recorded at 505 nm while the fibre was illuminated at 340 and 380 nm. Lower trace shows tension. Two tetani and two K^+ contractures were performed to obtain these records. Panel *B*, aequorin-injected fibre. Same experimental manoeuvres as in panel *A*. Upper trace shows aequorin light emission; lower trace shows tension.

In the records shown in Fig. 2, the peak ratio was approximately 2·8 and the resting ratio was 0·3. In ten preparations the peak ratio was $2{\cdot}4 \pm 0{\cdot}1$ and the resting ratio was $0{\cdot}33 \pm 0{\cdot}01$. These values have no absolute significance since they depend on the characteristics of the light source, filters, dichroic mirrors, etc. used in the experiments. However, they can be related to the properties of Fura-2 determined in our microscope with the same optical components. Since Fura-2 *in vitro* in our microscope has an R_{max} of ~ 8, it was important to discover whether the R_{max} in tetani of 2·4 is because the $[Ca^{2+}]_i$ is well below saturation or because the Fura-2 has changed its properties *in vivo*. Figure 4 shows an experiment designed to answer this point. Panel *B* compares the light signals produced by a tetanus and a K^+ contracture in a single fibre injected with aequorin (for details, see Allen *et al.* 1989*b*). The K^+ contracture produced about 5% more force than the tetanus and inactivated spontaneously despite the continued depolarization. The aequorin signal produced by the K^+ contracture was about 30 × greater than in the tetanus, suggesting that the $[Ca^{2+}]_i$ was about 4 × greater (since over this range light $\propto [Ca^{2+}]^{2{\cdot}5}$ for aequorin).

Figure 4*A* shows the results when the same procedure was performed using a Fura-2-injected preparation. Note that a presumably fourfold increase in $[Ca^{2+}]_i$ now only produced a very small increase in the peak 340 and 380 nm signals. The Fura-2 ratio was 1·42 in the tetanus and 1·66 in the K^+ contracture (this preparation had low ratios due to previous excess illumination). K^+ contractures were elicited in three preparations and the average increase in ratio as compared to a 50 Hz tetanus was 16% (range 5–26%).

Thus it appears that a fourfold increase in $[Ca^{2+}]_i$ (judged by aequorin) leads to only a 1·16-fold increase in Fura-2 ratio. It follows that Fura-2 is fairly close to saturation at the peak of a tetanus and, further, that the R_{max} of Fura-2 has changed from eight *in vitro* to approximately three *in vivo*. Similar changes have been observed in other tissues and have been attributed to the effects of intracellular viscosity on Fura-2 (e.g. Poenie, 1990).

Fura-2 records during fatiguing stimulation and during recovery

Figure 5 shows representative records of Fura-2 ratios and tension during fatiguing stimulation. The records illustrate four major findings: (i) there was a very small increase in peak Fura-2 ratio from the control (panel *a*) to the 10th (panel *b*) tetanus (peak ratio increased from 1·65 to 1·70); (ii) thereafter the peak ratio gradually declined reaching 1·45 in panel *f* when tension was 69% of control (50th tetanus) and 1·34 on the penultimate tetanus (57th tetanus, not shown) when tension was 45%; (iii) relaxation became slower as the fibre was fatigued and this slowing of relaxation was accompanied by a markedly reduced rate of decline of the Fura-2 ratio following each tetanus; and (iv) the resting ratio *before* each tetanus increased continuously during the period of fatiguing stimulation, which resulted in a much reduced difference between the peak ratio and the resting ratio in the fatigued state.

During fatiguing stimulation the peak ratios of the 10th tetanus compared with the first showed a small but significant increase of $5{\cdot}7 \pm 1{\cdot}8\%$ ($n = 6$, $P < 0{\cdot}05$). At this time the tension was reduced to $89 \pm 1\%$ of the control. Using aequorin we had previously found a marked increase (+55%) in the emitted light during this period (Allen *et al.* 1989*b*). In the aequorin study, tetanic light emission was also increased by exposing the fibres to 5 or 15% CO_2 and accordingly we ascribed the early increase

of light (and therefore also $[Ca^{2+}]_i$) to an intracellular acidification. Some of the fibres in the present study were also exposed to 5 or 15% CO_2 before fatiguing stimulation, but this exposure did not result in a measurable increase in peak ratio. However, because Fura-2 is approaching saturation at the peak of the tetanus as described

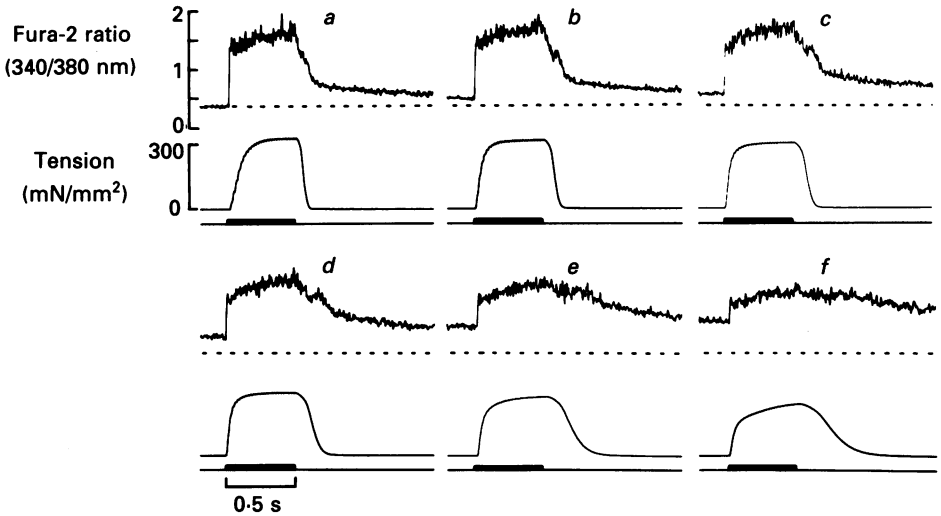

Fig. 5. Fura-2 ratio and tension recorded during fatigue caused by repeated intermittent tetani. Stimulation protocol as described in Methods. Panel *a*, control Fura-2 ratio and tension from a 0·5 s tetanus recorded at 2 min intervals before fatiguing stimulation. Panels *b–f*, subsequent panels show the 10th, 20th, 30th, 40th and 50th tetani during fatiguing stimulation. Dashed line on Fura-2 ratios indicates resting ratio in unfatigued fibre.

above, the expected increase of ratio would be small and correspondingly difficult to detect.

After the tenth tetanus, ratios started to decline. All fibres studied here were easily fatiguable (type 1, Westerblad & Lännergren, 1986) and the number of tetani needed to bring tension down to about 50% of control ranged from 36 to 100. In this fatigued state the peak ratio was reduced to 71 ± 4% of control ($n = 6$).

The tension records in Fig. 5 also show the well-known slowing of relaxation which is characteristic of fatigue. The Fura-2 ratios also show pronounced slowing of the phases of decline following a tetanus. The slope of phase I declined as fatigue progressed and phase II appeared later and became slower and more prominent. However, it still occurred at approximately the same time as the slow phase of tension decline accelerated into the exponential phase. Phase III did not obviously change its magnitude or time course when measured from the level preceding the tetanus. The net result of these changes was that by the end of fatiguing stimulation the Fura-2 ratio declined very slowly indeed. In fact, in some records (e.g. Fig. 5*f*) the decline of Fura-2 ratio which precedes relaxation is very small. We do not know whether this surprising finding reflects the $[Ca^{2+}]_i$ or if it arises from some artifact of the fura-2 signal, for instance movement artifacts which are not adequately corrected for by calculating the ratio.

Figure 6 shows original records obtained at the beginning and the end of fatiguing stimulation and during recovery. All four fibres which were followed during recovery displayed post-contractile depression; that is, a decline in tension occurred during the first part of the recovery period before tension finally recovered towards the

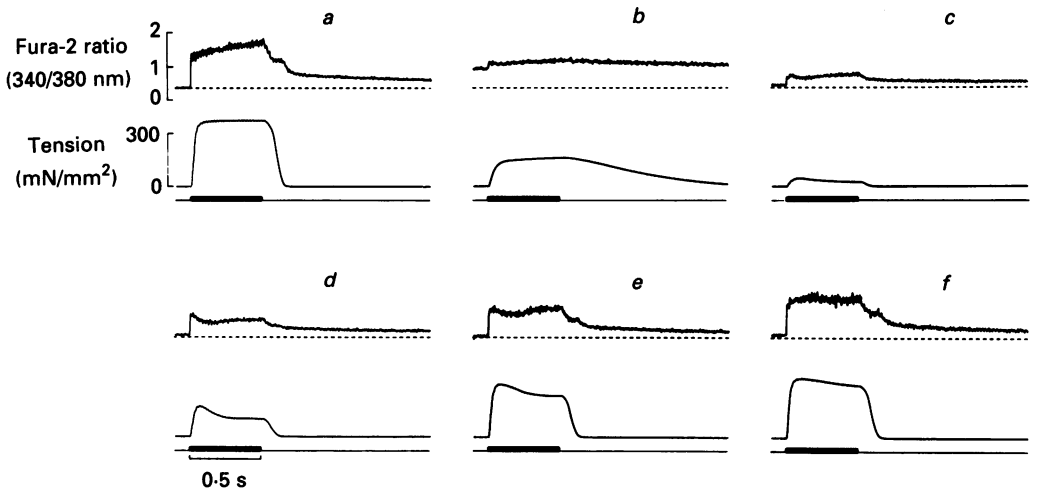

Fig. 6. Fura-2 ratio and tension during fatigue and subsequent recovery. Sample of individual records; fuller data shown in Fig. 7. Panels *a* and *b* show the control and 50th tetanus of a period of fatiguing stimulation. Panels *c*, *d*, *e* and *f* show tetani recorded 30, 60, 120 and 180 min after the end of fatiguing stimulation. Dashed line drawn through resting Fura-2 ratio in control.

control level (Westerblad & Lännergren, 1986). Post-contractile depression was accompanied by a further reduction of the peak ratio (Fig. 6*c*), which is in agreement with our previous results with aequorin (Allen *et al.* 1989*b*). After the post-contractile depression, both tension and peak ratio started to increase and after 3 h of recovery the tension and the peak ratio of the fibre in Fig. 6 had recovered to 94 and 85%, respectively, of the pre-fatigue control.

Figure 7 shows a plot of tension, peak Fura-2 ratios and resting Fura-2 ratios obtained from the fibre depicted in Fig. 6. During fatiguing stimulation, the resting ratio (the ratio immediately before each tetanus) increased continuously and at the end of the stimulation period it was 0·94, compared with 0·35 before fatiguing stimulation. This fibre was not stimulated during the first 30 min of recovery so that the recovery of the resting ratio could then be followed without any disturbance from test tetani. During the first minute of recovery the resting ratio diminished from 0·94 (the last value measured during fatiguing stimulation) to 0·76 (the first measured value during recovery which was 1 min after the last tetanus). The Fura-2 ratio then remained unchanged for 4 min before again starting to decrease and after 30 min it was almost back to control. The rapid recovery during the first minute was presumably the result of the recovery of the Fura-2 ratio from the influence of the immediately preceding tetani. The slower subsequent decline represents the reversal of some more long-term consequences of fatiguing stimulation.

Recovery of resting ratios was followed in six fibres and all of them recovered following a pattern similar to that described above; test tetani, which were produced in four of the six fibres, did not markedly affect the time course of recovery. At the end of fatiguing stimulation the ratio was 237 ± 16 % of the original and after 1 min,

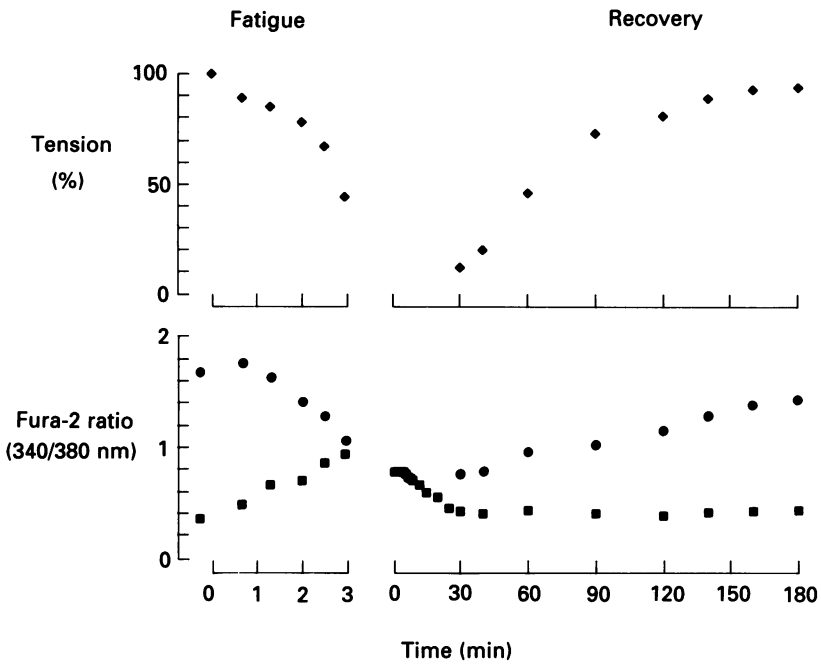

Fig. 7. Plot of tension and Fura-2 ratio throughout fatigue and recovery. Upper panel shows peak tetanic tension of tetani throughout fatiguing stimulation and recovery. Lower panel shows peak tetanic Fura-2 ratios (●) and resting Fura-2 ratios (■) during fatiguing stimulation and recovery. This muscle was not stimulated during the first 30 min of recovery so that the decline of resting Fura-2 ratios could be followed undisturbed by tetani. Same experiment as Fig. 6. The first measurements are for a control tetanus before fatiguing stimulation. The first measurement during recovery is 1 min after the last tetanus.

when the early component of decline was complete, the ratio had fallen to 198 ± 8 %. Thereafter recovery was much slower and it took about 30 min until the resting ratio was stabilized at 121 ± 2 % of the original.

Changes in Ca^{2+} sensitivity during fatigue

An interesting observation, seen in Figs 6 and 7, is that the *resting* Fura-2 ratio towards the end of fatiguing stimulation (Fig. 6, panel *b*) is greater than some of the *tetanic* Fura-2 ratios during the early part of recovery (Fig. 6, panels *c* and *d*). Similar results were observed in all four muscle fibres in which this comparison could be made. This suggests that the Ca^{2+} sensitivity of the fibres was substantially reduced towards the end of fatiguing stimulation.

To explore this possibility further we have plotted the peak tension and peak Fura-2 ratios as $[Ca^{2+}]_i$–tension curves (Fig. 8). In our previous study with aequorin

(Allen *et al.* 1989*b*), *in vivo* $[Ca^{2+}]_i$–tension curves were constructed from K^+ contractures produced before fatiguing stimulation. This approach could not be used in the present studies because the Fura-2 ratios were obtained from two successive contractions and, especially at low K^+ concentrations, two identical contractures

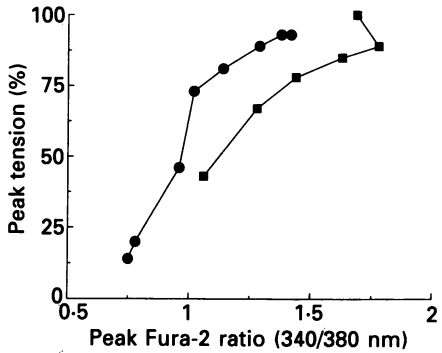

Fig. 8. Plot of peak tetanic tension against peak Fura-2 ratio throughout fatigue (■) and recovery (●). Same experiment as Figs 6 and 7.

could not be produced. Instead we have made use of the delayed tension recovery after fatiguing stimulation to construct Fura-2 ratio–tension curves. If the first recovery value is taken after at least 20 min the intracellular pH (pH_i) has recovered (Westerblad & Lännergren, 1988) and since phosphorus metabolites probably recover faster than pH_i (Kushmerick & Meyer, 1985), the recovery values should not be influenced by metabolic factors known to reduce the $[Ca^{2+}]_i$ sensitivity and the maximum tension-generating capacity of the contractile proteins (Fabiato & Fabiato, 1978; Kentish, 1986; Godt & Nosek, 1989). Furthermore, in the aequorin study we found that the values obtained during recovery lie very close to the unfatigued $[Ca^{2+}]_i$–tension curves, and we have also recently shown a uniform distribution of $[Ca^{2+}]_i$ in recovery tetani (Westerblad, Lee, Lamb, Bolsover & Allen, 1990). Thus, it seems that the normal relation between peak ratio and tension can be obtained from the recovery values.

In Fig. 8 such a Fura-2 ratio–tension curve has been constructed from recovery values (●) obtained from the fibre in Figs 6 and 7. It can be seen that the values obtained during fatiguing stimulation (■) always lie to the right of this curve, which suggests a reduced Ca^{2+} sensitivity. This reduction reaches a maximum during the first part of the stimulation period where the peak ratio is slightly increased and the tension is decreased. The values thereafter return towards the control curve but still lie to the right of the recovery values. Similar results were obtained in three of the four muscle fibres which could be examined in this way. In the other muscle fibre there was no consistent shift between recovery and fatigue.

Mechanism of elevated resting $[Ca^{2+}]_i$ *during fatigue*

The results described above show that resting $[Ca^{2+}]_i$ gradually increases during fatiguing stimulation and then slowly recovers over a period of approximately 30 min. It is known that fibres become depolarized during fatigue and also that there

is an intracellular acidosis. Both of these recover within 20–30 min after fatigue and both are known to raise intracellular $[Ca^{2+}]_i$ (for references, see Discussion). We therefore attempted to assess the contribution that these changes made to the elevated resting $[Ca^{2+}]_i$ during fatigue.

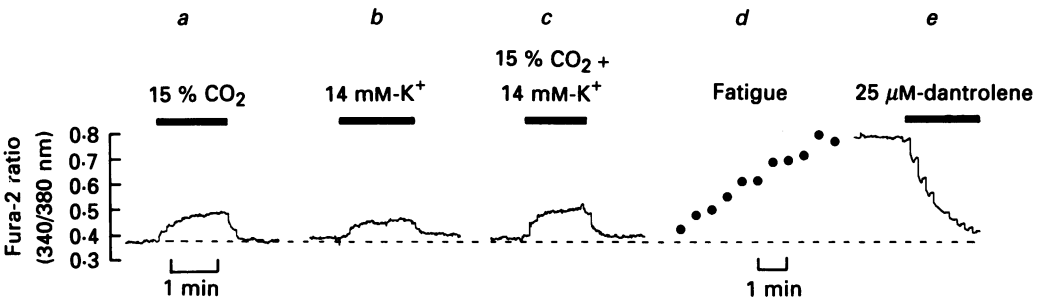

Fig. 9. Effect of various interventions on resting Fura-2 ratios: panel *a*, 15% CO_2 equilibrated with Ringer solution (pH = 5·9). Panel *b*, K^+ in Ringer solution increased from 2·5 to 14 mM. Panel *c*, combined effect of 15% CO_2 and 14 mM-K^+. Panel *d*, resting Fura-2 ratios (measured immediately before tetani) during fatiguing stimulation. Note time scale in this panel is different to the others. Panel *e*, 1·5 min after the end of the fatiguing stimulation 25 μM-dantrolene was applied.

Figure 9 illustrates the approach used. Resting $[Ca^{2+}]_i$ was monitored during application of Ringer solution saturated with 15% CO_2 (panel *a*), a concentration chosen because it produces an intracellular acidosis similar to that during fatigue (see Discussion). When resting $[Ca^{2+}]_i$ had recovered after exposure to CO_2, $[K^+]_o$ was increased from 2·5 to 10 mM (not shown) and 14 mM. These concentrations of K^+ would cause a depolarization slightly larger than that observed in fatigue (see Discussion). Figure 9 (panel *b*) shows the application of 14 mM-K^+ which produced a substantial increase in $[Ca^{2+}]_i$. Since in fatigue the acidosis and the depolarization occur simultaneously a solution containing both 15% CO_2 and 14 mM-K^+ was then applied. This produced a rise in $[Ca^{2+}]_i$ which was only slightly greater than the larger of the two previous responses (panel *c*). The same muscle fibre was then stimulated until fatigued and the points in panel *d* indicate the gradual rise in $[Ca^{2+}]_i$ between tetani. Note that the rise in $[Ca^{2+}]_i$ during fatigue was much greater than that produced by acidosis and depolarization.

Similar experiments were carried out in six preparations. In these muscle fibres the resting Fura-2 ratio was $0{\cdot}33 \pm 0{\cdot}01$. Fifteen per cent CO_2 caused a rise of $0{\cdot}08 \pm 0{\cdot}01$ and 10 mM-K^+ caused a rise of $0{\cdot}06 \pm 0{\cdot}02$ units. In three of these preparations simultaneous application of CO_2 and K^+ produced effects which were similar though slightly larger than the largest of the responses to either intervention applied individually. A satisfactory fatigue run was completed in three of these preparations and increased the resting ratio by 0·33 units. Taking all the fatigue runs available to us ($n = 6$), the mean increase in resting Fura-2 ratio was $0{\cdot}50 \pm 0{\cdot}07$ units, which is clearly much greater than that caused by either acidification or depolarization or both together.

Three muscle fibres were exposed to dantrolene (25 μM) shortly after fatiguing

stimulation when the Fura-2 ratio was still elevated. Figure 9*e* shows one example in which dantrolene was applied 1·5 min after the last tetanus of a fatigue run. During the following 2 min the resting ratio rapidly declined from 210 to 115% of the control; during this period the resting ratio was normally virtually unchanged (see above). Similar results were obtained in two other fibres exposed to dantrolene during the recovery. Dantrolene exposure of rested fibres caused only a marginal reduction of the resting Fura-2 ratios.

DISCUSSION

Changes in $[Ca^{2+}]_i$ during fatigue

In our previous study of $[Ca^{2+}]_i$ during fatigue using aequorin, we found that the tetanic aequorin signal rose to 155% of control in the early part of fatigue and then declined to 15% by the time tension had fallen to 50%. In the present study, the Fura-2 ratio rose to 106% early in fatigue and then declined to 71%. How similar are the changes in $[Ca^{2+}]_i$ which these two indicators are reporting? These issues are explored in Fig. 10. Panel *A* shows the relation between aequorin light emission and $[Ca^{2+}]$ under conditions thought to resemble the intracellular environment (for details see figure legend). The peak aequorin light during a tetanus represents a $[Ca^{2+}]_i$ of 5·2 μM; the 55% increase in the early part of fatigue represents a 19% increase in $[Ca^{2+}]_i$, while the decrease to 15% when tension had fallen to 50% represents a fall in $[Ca^{2+}]$ to 46% of the control level. Panel *B* shows the relation between Fura-2 ratio and $[Ca^{2+}]_i$; note that its properties are very different to aequorin, close to saturation for tetanic $[Ca^{2+}]$ but very sensitive at low $[Ca^{2+}]_i$. For the present purpose we have assumed that $[Ca^{2+}]$ determined by aequorin is correct and chosen a K_d for Fura-2 which matches the measured Fura-2 ratio to the $[Ca^{2+}]_i$. The Fura-2 obtained in this way is 138 nM, which compares with previous estimates in muscle of 88 nM (Klein *et al.* 1988) and 230 nM (Baylor & Hollingworth, 1988). The 6% increase in Fura-2 ratio represents a 44% increase in $[Ca^{2+}]_i$, while the decrease to 71%, when tension had fallen to 50%, represents a fall in $[Ca^{2+}]_i$ to 29%. Thus the two indicators report roughly similar changes in $[Ca^{2+}]_i$ at the various stages of fatigue. Since both indicators also report slowing of the decline of tetanic $[Ca^{2+}]_i$ during fatigue, a rise in resting $[Ca^{2+}]_i$ and qualitatively similar changes during recovery, the degree of agreement between these two very different indicators seems satisfactory.

Slowing of relaxation during fatigue

The present results have confirmed the finding of Cannell (1986) that complex changes in $[Ca^{2+}]_i$ occur during the relaxation of unfatigued muscle fibres. The early rapid decline (phase I), which Cannell attributed to rapid uptake of Ca^{2+} by both parvalbumin and the sarcoplasmic reticulum, showed a substantial slowing during fatiguing stimulation. The extent to which this slowing is due to parvalbumin saturation or to a reduced rate of sarcoplasmic reticulum uptake is not clear from the present experiments. The transient reversal of $[Ca^{2+}]_i$ decline (phase II) was attributed by Cannell to release of Ca^{2+} from troponin when cross-bridges suddenly became detached. This phase also became slower and delayed in fatigue, consistent

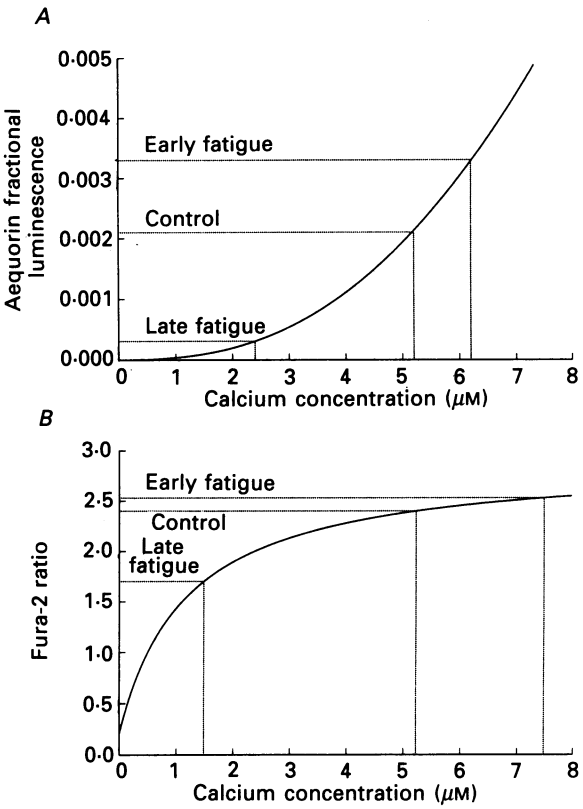

Fig. 10. Comparison of changes in $[Ca^{2+}]_i$ during fatigue as estimated using aequorin and Fura-2. Panel *A*, relation between aequorin light emission and $[Ca^{2+}]_i$. The line shows fractional luminescence (L) where $L = [(1+3{\cdot}5\times10^6[Ca^{2+}])/(1+130+3{\cdot}5\times10^6[Ca^{2+}])]^3$, which fits *in vitro* calibration curves in 150 mM-K^+, 1 mM-Mg^{2+}, pH 7·0, 21 °C (see Allen, Blinks & Prendergast (1977) for further details). The fractional luminescence for the peak aequorin light from unfatigued tetani (control) was determined from data in Allen *et al.* (1989*b*) and was $2{\cdot}1\pm0{\cdot}3\times10^{-3}$ ($n = 7$) (for details see Allen & Blinks, 1979). This gives an estimate of peak tetanic $[Ca^{2+}]_i$ of 5·2 μM. The other dashed lines indicate the peak tetanic luminescence when the maximum increase had occurred early in fatigue (early fatigue) and the peak tetanic luminescence when tension had declined to 50% of control (late fatigue). Panel *B*, relation between Fura-2 ratio and $[Ca^{2+}]_i$ from Grynkiewicz *et al.* (1985). The line shows the solution to

$$[Ca^{2+}] = K_d\left[\frac{R-R_{min}}{R_{max}-R}\right]\frac{S_{f2}}{S_{b2}}, \quad (1)$$

with $R_{max} = 2{\cdot}9$, $R_{min} = 0{\cdot}20$, $S_{f2}/S_{b2} = 8{\cdot}6$ and $K_d = 138$ nM. R_{max} was determined on the following basis. Equation (1) can be simplified to

$$[Ca^{2+}] = K\frac{R'}{1-R'}, \quad (2)$$

where $R' = (R-R_{min})/(R_{max}-R_{min})$ i.e. R' is the fractional value of R between R_{min} and R_{max} and K is the apparent dissociation constant. For our data we know that a fourfold increase in $[Ca^{2+}]$ above the tetanic level leads to a 1·16-fold increase in R. Thus, if the increase in $[Ca^{2+}] = a$ and in $R = b$ we have, to a good approximation,

$$a[Ca^{2+}] = K\frac{bR'}{1-br'}. \quad (3)$$

with the slow rate of cross-bridge cycling observed in fatigue (Lännergren & Westerblad, 1989). If phase II originates from detaching cross-bridges, it should not be detected in fibres stretched to sarcomere lengths $> 3{\cdot}6\ \mu m$. It is of interest therefore that phase II is not detectable in the studies of Baylor & Hollingworth (1988) or Klein *et al.* (1988) in which the fibres were stretched to $> 3{\cdot}6\ \mu m$.

A surprising finding was that in the later part of fatigue, relaxation appeared to occur when the $[Ca^{2+}]_i$ had shown little decline (e.g. Fig. 5*f*). No such phenomenon was observed in our aequorin study (which is insensitive to movement artifacts) and the influence of movement artifacts during this phase of $[Ca^{2+}]_i$ need to be excluded before this observation is given greater weight.

The relation between tetanic tension and $[Ca^{2+}]_i$ in fatigued fibres

In our previous study with aequorin (Allen *et al.* 1989*b*), it was concluded that reduced tetanic $[Ca^{2+}]_i$ could explain all of the tension reduction in fatigued fibres (tension reduced to approximately 50%) and that the Ca^{2+} sensitivity of the contractile proteins at this stage of fatigue appeared to be normal. The latter finding conflicts with results from skinned muscle fibres where metabolic changes known to accompany fatigue have been found to cause a reduction in the Ca^{2+} sensitivity (e.g. Godt & Nosek, 1989). The present results with Fura-2 also suggest that a reduction in Ca^{2+} sensitivity in living muscle fibres may contribute to fatigue because (i) values obtained in the fatigued state lay to the right of the constructed ratio–tension curves and (ii) the resting ratios in the fatigued state were sometimes higher than the peak ratios during recovery. Since peak ratios at the end of fatiguing stimulation are in the range where the ratio–tension curves are steep (see Fig. 8), a small change of the ratio will cause a large change in tension. Accordingly the relatively small rightward shift of peak ratios in the fatigued state could explain up to half of the tension decline. This finding conflicts with the results from our previous study with aequorin where no reduction in Ca^{2+} sensitivity was seen although experiments designed to reveal such an effect were performed. One possibility is that the intracellular concentration of Mg^{2+} may increase during fatigue (cf. Irving, Maylie, Sizto & Chandler, 1989) and this would reduce the aequorin light at a given $[Ca^{2+}]_i$ and hence disguise a fall in Ca^{2+} sensitivity. Assessment of this hypothesis requires measurements of $[Mg^{2+}]_i$ during fatigue.

Increased resting $[Ca^{2+}]_i$ in fatigued fibres

This study shows that the resting $[Ca^{2+}]_i$ increased markedly during fatiguing stimulation and the recovery of this increase took 20–30 min. Part of this rise in resting $[Ca^{2+}]_i$ could occur through summation of the tails of $[Ca^{2+}]_i$ after each tetanus (phase III). We think this contribution is small because measurements of

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Simplifying eqns (2) and (3), $R' = (a-b)/(ab-b)$ and for the numerical values above $R' = 0{\cdot}82$. Since $R = 2{\cdot}4$, R_{max} is approximately $2{\cdot}9$; the value depends only slightly on the estimate of R_{min} since this is relatively small. The value of R_{min} was chosen fairly arbitrarily; the only information we have is that is should be close to or less than $0{\cdot}22$, the value obtained in one experiment when EGTA was microinjected into the fibre. The uncertainty about R_{min} means that estimates of resting $[Ca^{2+}]_i$ from the measured resting R are unreliable. S_{f2}/S_{b2} was measured in our apparatus. K_d was chosen so that tetanic $[Ca^{2+}]_i$ under control conditions was $5{\cdot}2\ \mu M$, as determined by aequorin. Other lines are as defined in panel *A*.

$[Ca^{2+}]_i$ after 1 min of recovery, when the tails of $[Ca^{2+}]_i$ should have decayed, show that $[Ca^{2+}]_i$ was still substantially elevated. There are many other possible causes for such a change in resting $[Ca^{2+}]_i$ and we have considered three possibilities: (a) an intracellular acidification, (b) a depolarization and (c) changes in the Ca^{2+} handling of the SR.

(a) An acidification of about 0·6 pH units develops during fatiguing stimulation of the type used in this study and recovers with a time course similar to that of the resting ratio (Westerblad & Lännergren, 1988). Application of 15% CO_2 can be estimated to produce an acidification of 0·45–0·75 pH units (Bolton & Vaughan-Jones, 1977; Curtin, 1987). We found that application of 15% CO_2 caused a small increase in resting $[Ca^{2+}]_i$, as has previously been noted in barnacle skeletal muscle (Lea & Ashley, 1978) and in mammalian cardiac muscles (Bers & Ellis, 1982). The mechanism that underlies this interaction between H^+ and Ca^{2+} has not been identified; what is clear, however, is that the observed increase in $[Ca^{2+}]_i$ due to acidosis can only explain a small fraction of the observed resting $[Ca^{2+}]_i$ increase in fatigue.

(b) During fatiguing stimulation fibres become depolarized to about −70 mV (Westerblad & Lännergren, 1986) and the recovery of this depolarization has a time course similar to that of the resting Fura-2 ratio. Small depolarizations have previously been shown to elevate the resting $[Ca^{2+}]_i$ in muscle fibres (López, Alamo, Caputo, DiPolo & Vergara, 1983; Snowdowne, 1985), probably by increasing the leakage of Ca^{2+} from the SR. In the rested fibres exposed to 10 mM-KCl, which would result in a depolarization to about −65 mV (Hodgkin & Horowicz, 1959), the resting ratio increased but this could explain only a small fraction of the observed increase in fatigued fibres.

(c) Dantrolene is thought to reduce the $[Ca^{2+}]_i$ leakage from the SR by closing the SR Ca^{2+} channels (Hainaut & Desmedt, 1974; Suarez-Isla, Orozco, Heller & Froehlich, 1986). The rapid decline in resting Fura-2 ratio when dantrolene was applied could be caused by two distinct mechanisms. (i) The SR may have been excessively leaky during fatigue and this leakiness is reversed by dantrolene. (ii) The SR leak may be normal during fatigue but the Ca^{2+} uptake by the SR pump may be greatly reduced. Dantrolene may be capable of reducing Ca^{2+} efflux from SR to below the normal level and thus balancing influx and efflux at a lower $[Ca^{2+}]_i$. Thus, the dantrolene experiments suggest that Ca^{2+} cycling between SR and myoplasm is abnormal during fatigue but cannot distinguish between an increased SR Ca^{2+} leakage and a reduced SR Ca^{2+} uptake. However, the fact that dantrolene could return the resting $[Ca^{2+}]_i$ rapidly to close-to-normal levels suggests that changes in SR function have a major role in the increased $[Ca^{2+}]_i$ during fatigue.

A prolonged increase of the resting $[Ca^{2+}]_i$ will increase the muscle metabolism (e.g. Solandt, 1936; Hill & Howarth, 1957; Snowdowne, 1985). Further, it may stimulate protease activity (Duncan, 1978; Turner, Westwood, Regen & Steinhardt, 1988) and cause energy uncoupling and structural damage of mitochondria (for review, see Chapman & Tunstall, 1987). In the present context it is tempting to speculate whether the rise in resting $[Ca^{2+}]_i$ might be involved in the reduced tetanic $[Ca^{2+}]_i$ levels during post-contractile depression. Several lines of evidence suggest that post-contractile depression is caused by a failure of transmission of signals between the T-

tubules and the SR (e.g. Lännergren & Westerblad, 1989). We therefore suggest that a prolonged elevation of $[Ca^{2+}]_i$ might cause impaired function of proteins involved in the signal transmission between the T-tubules and the SR. Thus, the prolonged elevation of rested $[Ca^{2+}]_i$ during fatiguing stimulation and recovery may be of considerable physiological interest.

In summary, we have shown that the tension reduction in *Xenopus* muscle fibres fatigued by repeated tetani is caused by a combination of reduced tetanic $[Ca^{2+}]_i$ and reduced $[Ca^{2+}]_i$ sensitivity. Further, the resting $[Ca^{2+}]_i$ increased markedly during fatiguing stimulation and it remained elevated long into recovery.

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